Characterisation of the efficacy of endodontic medications using a three-dimensional fluorescent tooth model: An *ex vivo* study

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Abstract

The purpose of this study was to establish a three-dimensional fluorescent tooth model to investigate bacterial viability against intra-canal medicaments across the thickness and surface of root dentine. Dental microbial biofilms (Enterococcus faecalis and Streptococcus mutans) were established on the external root surface and bacterial kill was monitored over time against intra-canal medicament (Ca(OH)2) using fluorescent microscopy in conjunction with BacLight SYTO9 and propidium iodide stains. An Olympus digital camera fitted to SZX16 fluorescent microscope captured images of bacterial cells in biofilms on the external root surface. Viability of biofilm was measured by calculating the total pixel area of green (viable bacteria) and red (non-viable bacteria) for each image using ImageJ® software. All data generated were assessed for normality and then analysed using a Mann–Whitney t-test. The viability of

S. mutans biofilm following Ca(OH)2 treatment showed a significant decline compared with the untreated group (P = 0.0418). No significant difference was seen for E. faecalis biofilm between the Ca(OH)2 and untreated groups indicat- ing Ca(OH)2 medicament is ineffective against E. faecalis biofilm. This novel three-dimensional fluorescent biofilm model provides a new clinically relevant tool for testing of medicaments against dental biofilms.

Keywords

bacterial infection, biofilm, calcium hydroxide, endodontics, Streptococcus mutans.

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Introduction

The understanding of microbial ecology and bacterial fitness dynamics within the root canal is fundamental to the management of endodontic disease (1). The success of endodontic treatment lies in the elimination of bac- teria and their by-products from the canal spaces, as well as the establishment of a complete seal (2,3). Bacterial biofilms within the root canal are a major challenge for endodontic therapy because they protect microorganisms from adverse environmental challenges including antimicrobial action (1,4). Re-infection of endodontically treated teeth may stem from persist- ence of bacterial biofilms in unprepared locations including in the main canal, isthmuses and accessory canals, thus threatening the success of endodontic therapy (5–7).

Several models have been used for evaluating the effi- cacy of medications for root canal therapy (8–11). Each of these methods has provided insight into the efficacy of various treatments and conditions that influence bacterial viability on teeth. Past models such as the simple plate count method are limited in that it cannot replicate the biological complexity of bacterial biofilms in which there is continual cell growth and death, and persistent cell turnover (4,12,13). The frequently used agar diffusion test is dependent on the ability of the medications to diffuse in agar (8). Calcium hydroxide's poor diffusion into agar and the buffering action of agar makes this model unsuitable for testing its efficacy (8,14–16). The use of scanning electron microscopy (SEM) as a possible method to evaluate efficacy of medicament (9,10,17,18) is limited by not only the susceptibility of bacteria to its preparation techniques but also its inability to visualise all sections of teeth with hidden microorganisms (11). Dentine block model, created by Haapasalo and Orstavik (10,11), involves the sealing of one end of the dentinal tubules with nail polish, which prevents the evaluation efficacy of medicament across tubules. The use of dentine scrapings to evaluate disinfection on selected walls of the root canal does not provide a three-dimensional profile of disinfection along the length and thickness of the root dentine (19).

The viability of microorganisms may vary throughout tooth surface biofilms (20). Ma et al. proposed a three- dimensional model that allowed for quantitative assess- ment of bacterial viability for short-term disinfection in sectioned roots by the use of irrigants against predictable, dense and deep-penetrating bacteria (21). This model however does not allow for assessing the uniformity of bacterial kill of medicaments over time points or the survival dynamic of the bacterial biofilms over a time period; further, the study design allowed for evaluating only a small section of the root dentine.

The aim of the current study was to establish an ex vivo model for evaluating the uniformity of bacterial kill in dental bacterial biofilm by medicaments diffusing across dentinal tubules to the external root surface, over an extended time period of 72 h. This model assessed the efficacy of treatment using a dynamic E. faecalis and S. mutans biofilm grown on the external root surface rather than within the root canal.

Materials and methods

Sample preparation

Thirty-two single-rooted extracted human teeth with intact crowns were collected from adult patients attend- ing dental clinics. This study was approved by the Griffith University Human Research Ethics Committee. Prior to commencing this study, all crown and root sur- faces of each tooth were debrided with an ultrasonic scaler (Satelec/Acteon, Niort, France) to remove residual soft tissue and calculus and then sterilised in an auto- clave (121°C for 15 min). A sterile carbide bur was used to establish access to the pulp chamber and canals. The working length of each tooth was established 1 mm short of the apical foramen by inserting a size 10 K-file (Dentsply, Maillefer, Ballaigues, Switzerland) past the apex until the tip was visible, and then withdrawing the file by 1 mm. ProTaperTM files (Dentsply) were used to enlarge the root canals. All canals were prepared using ProTaperTM files (Dentsply) according to manufacturer's instruction to a final file size corresponding to an F3 instrument. The canals were copiously irrigated alterna- tively with 1% NaOCl (Dentalife, Ringwood, Victoria, Australia) and 15% EDTA/C (EDTA/C 15%; Endoprep solution; Bayswater, Victoria, Australia, Milton's solu- tion) during the mechanical preparation. The prepara- tion was followed with a rinse of NaOCl for 2 min and EDTA/C for 2 min to ensure complete removal of smear layer and finally flushed with de-ionised water.

Establishment of biofilm on the surface of the root

To prepare the model, a hole was initially bored into a coverlid of a sterile cylindrical 5 mL vial. The tooth was then inserted into the lid to the cementoenamel junction (CEJ) and stabilised and sealed with sticky wax (Kemdent, Brisbane, Queensland, Australia). The set-up enables the crown of the tooth to protrude above the lid, and the root of the tooth to be suspended in the centre of the vial (Fig. 1). To prevent bacteria from the culture broth entering the canals through the apex of the roots, the apex of the each tooth was sealed with wax, and a paper point was placed into the chemo-mechanically pre- pared canal to indicate effectiveness of the seal; a moist paper point would indicate a lack of proper seal.

To support biofilm growth a salivary pellicle was first generated around the surface of the root by submerging teeth in fresh, unstimulated saliva, pooled from two healthy volunteers, up to the level of the CEJ for 24 h at 37°C statically (18). Teeth with salivary pellicle were then submerged into 5 mL of freshly diluted Todd Hewitt broth (THB; 1/10 in water) with inoculate of 5 mL of an over- night broth culture of S. mutans or E. faecalis. The diluted broth was changed every 2 days to ensure the correct pH and nutrient levels were sustained. The broth was used in diluted form as

to create a nutrient poor environment, therefore encouraging biofilm formation rather than planktonic growth. The S. mutans and E. faecalis used in this study were originally isolated at the University of Alabama, Birmingham Hospital (22,23), and were grown in THB (Oxoid, North Ryde, New South Wales, Australia) for 18 h at 37°C shaking at 180 rpm. The teeth set-up in the vials was incubated at 37°C to mimic the oral environ- ment. At 48 h, fresh media were added by removing three-quarters of the original suspension from each vial and replacing this with fresh, diluted THB. Preliminary experiments demonstrated that establishment of S. mutans or E. faecalis surface biofilm on the root required 4 days, which we regarded as time zero; medicament was then applied at this time point (t = 0 h).

Experimental groups

Once biofilm formation was confirmed, each experimen- tal root canal was treated as per Table 1. Paper points were removed from each of the sample teeth. The root canal of each sample tooth of the positive control groups (n = 2 × 7 for S. mutans and E. faecalis) was sealed at the coronal part of the tooth with cavit G (3M, North Ryde, New South Wales, Australia). For the calcium hydroxide (Ca(OH)2) treatment groups (n = 9 each for S. mutans and E. faecalis), calcium hydroxide paste (Henry Schein, Melville, NY, America) was placed in the root canal and sealed with Cavit G®.

To analyse bacterial survival dynamics following treat- ment with calcium hydroxide on the surface of biofilm, we captured representative images at 0, 1, 3, 6, 24, 48 and 72 h of Baclight stained teeth/biofilms (see below). Between each time point, it was ensured that each tooth root was fully submerged in phosphate-buffered saline (PBS) in order to prevent the bacterial biofilm on the root surface from drying out.

Bacteria and fluorescent staining techniques

For staining of bacterial biofilms, teeth were submerged in 3 mL of BacLight stain for 15 min in the dark (Viable Technologies, Mulgrave, Victoria, Australia). The stain is composed of SYTO9, which stains viable bacteria (emit- ting green fluorescence), and propidium iodine, which stains non-viable bacteria (emitting red fluorescence) and was prepared according to the manufacturer's instruc- tions (24). The excitation/emission wavelengths for SYTO9 were 480/500 nm and for propidium iodide 490/ 635 nm, with GFPHQ and RFP1 filters used, respectively. The teeth were immersed briefly in a PBS series following BacLight staining to remove residual stain. The staining procedure was repeated before each time point prior to microscopic analysis.

For visualisation of viable and non-viable bacterial cells in biofilms, images were acquired using a DP72 Olympus digital camera fitted to an Olympus three-

dimensional SZX16 fluorescent microscope (Olympus, Center Valley, PA, USA). Viability of the biofilm was measured by calcu- lating the total pixel area of green (viable bacteria) and red (non-viable bacteria) for each image. ImageJ 1.45s software (National Institutes of Health, Bethesda, Mary- land, USA) was used to measure pixel densities for the green and red channels in each image. All data generated were assessed for normality using Kolmogorov–Smirnov test and then analysed using unpaired Mann–Whitney t-test. Scanning electron microscopic images of an untreated tooth with E. faecalis biofilm was obtained (at zero time point) to visualise bacterial presence. The image was obtained using a Zeiss Sigma VP field emission scanning electron microscope at low vacuum. Further, presence of bacterial and bacterial kill was visualised following development of E. faecalis bacterial biofilm on the surface of the tooth using higher resolution fluorescent images that were acquired with a Nikon A1R (Nikon Corporation, Chiyoda-ku, Tokyo, Japan) laser scanning confocal microscope; fluorescent dyes were as above.

Results

This study established a successful three-dimensional biofilm model on the external surface of the root as a means to examine the efficacy of medicament treatment to the root canal (Fig. 1). Firstly, to establish that the model of biofilm formation was indeed occurring SEM was performed to obtain high-resolution images of the tooth surface. This demonstrated the formation of extracellular matrix, consistent with biofilm formation and bound coccoid bacteria on the surface of the root (Fig. 2, black arrow). The surface of the tooth can also be seen in the top left of the image. This was further con- firmed with fluorescent imaging using laser scanning confocal microscopy (Fig. 3a). Fluorescent imaging dis- played clusters of both live (green) and dead (red) bacte- ria (Figs 3b and 2c).

Once the model of biofilm formation on the external surface of the root was confirmed, we used the three- dimensional model to examine the efficacy of medica- ments, applying Ca(OH)2 to the root canal of the tooth and staining S. mutans and E. faecalis biofilms for viability on the external root surface. This demonstrated viability and death of the bacterial biofilms on the surface of the roots over 72 h and their growth patterns when subjected to Ca(OH)2 treatment (Figs 4 and 5). The control groups showed that the growth of both S. mutans and E. faecalis biofilm were unstable in the first few hours (viability of both types of biofilms decreased sharply at 3 h); however, both control sample biofilm models tested in this study showed consistent viability after 3 h (Fig. 4).

Following the first 3 h of Ca(OH)2 treatment, the viabil- ity of E. faecalis biofilm showed a gradual decrease when compared with the control group for the first 24 h; however, this was not statistically significant. The viabil- ity of the E. faecalis biofilm after 24 h showed gradual increases, reaching to a similar level of biofilm viability as the control group over 72 h (Fig. 4a).

S. mutans biofilm in the Ca(OH)2 treatment group exhibited a decrease in viability in the first 6 h. The viabil- ity continued to decrease over time with significant dif- ferences ($P = 0.042$) being noticed to the control group at 48 h and the end of the study period (72 h; Fig. 4b). This is further demonstrated in Figure 5 where the red stain- ing is in greater proportion to the green in panels A, B and C compared with the greater intensity of green in panels D, E and F. This indicates that the application of Ca(OH)2 into the root canal is able to kill S. mutans on the external root surface and demonstrates the power this model of biofilm formation has on its ability to expand this field of research.

Discussion

Current approaches to study the efficacy of medicament towards surface bacterial biofilms on teeth are largely based on those involving agar diffusion (8,14,15), infected human and bovine teeth ex vivo using microscopy (9,10) and are limited in efficacy of medicaments and assessing three-dimensional penetration. Thus, it is important to determine if medicaments can penetrate to sufficient depth into the dentinal tubules and effectively reach the external surface to be effectual against bacterial biofilms. The major disadvantage of the other models is the tooth samples were to be sacrificed, or were limited to being assessed as only scrapings or smears, which makes real-time evaluation in three-dimension impossible (8,14,15).

Endodontic bacteria can be detected at various depths and can exist as single units or as biofilm aggregates within the tubular structure of dentine (25,26). The present study used a unique ex vivo model external root surface model to grow bacterial biofilm, with the purpose of evaluating the efficacy and uniformity of bacterial kill of intra-canal medicament following the diffusion through thickness of the complex dentinal tubular structure. This model also allows for evaluating of survival dynamics of biofilms in real time over a period of time (72 h) on the tooth root.

Calcium hydroxide has been considered the gold standard for medication of the root canal (27). Calcium hydroxide is a non-staining intra-canal medicament that is reported to be difficult to remove; however, the strong alkalising effects of Ca(OH)2 stemming from the generation of hydroxyl ions are directly antibacterial and have broad-spectrum activity against different microbes (27– 29). Calcium hydroxide can diffuse up to 500 μm into dentinal tubules and is reported to more than halve the viable bacteria within 7 days (30). Clinical studies of the efficacy of Ca(OH)2 on previously untreated bacterial biofilms have shown more than 90% elimination of bacteria (31,32). Studies have revealed that hydroxyl ions derived from Ca(OH)2 diffuse through root dentine and have greater penetration into biofilm (9,33).

The microorganism species seen in infected root canals can vary depending on the clinical conditions (34). E. faecalis is commonly associated with failure of root canal treatment (11,35–38); however, Ca(OH)2 is not known to be effective against E. faecalis biofilms (39). In our study, Ca(OH)2 was used as the prototypic medicament for testing our model of both E. faecalis and S. mutans dental biofilm, as the former is a recognised bacteria seen in infected root canals, while the latter is known to be susceptible to Ca(OH)2 and helps provide validity to this model (38,40). The present study showed there was an increase in viability of bacterial biofilms from hour 3, which peaked at hour 24 and remained stable for both strains of bacterial biofilms. This trend persisted for both bacterial biofilms and subsequently decreased for the remainder of the assay as broth was not replenished during the 72 h. The initial decrease in viabil- ity of bacterial biofilms on the surface of teeth (at

hours 0, 1 and 3) could be attributed to constant handling and disturbance to the bacterial biofilms (Fig. 4). This was controlled for to some extent through the imaging of one surface of the tooth, so that only the opposite side was disturbed during the imaging process.

This study model demonstrated, for the first time, a notably different survival dynamics of S. mutans and E. faecalis biofilms on teeth following Ca(OH)2 treatment compared with the overall root surface. It is important to note that the treatment groups were compared with the control group to demonstrate validity of the model. Thus, this model offers a unique advantage of allowing for monitoring the survival dynamics of dental biofilms in response to different intra-canal medication.

Root surface images captured prior to bacterial staining revealed either auto-fluorescence of teeth or fluorescence of the epithelial cells with the SYTO9 stain from the salivary pellicle (41). Consequently, the results may have shown an underrepresentation of the amount of bacterial kill. A counter stain that has no reaction with the viability of the bacterial biofilm or sterifiltration of the saliva may be required to minimise green fluorescence of the tooth. As samples were reused for each time point, the fluorescent stains may have influences on the viability of bacteria. It may also be possible with the use of advanced fluorescence micro-scopes to evaluate the amount of auto-fluorescence's and hence enabling better evaluation of the bacterial cells using the BacLight live/dead stain. An additional advantage of this model is that the external root surface of the teeth can be ground to different fixed dimensions from the internal root canal wall to standardise the assessment of depth of kill and uniformity of kill over different time points.

Conclusion

The novel three-dimensional fluorescent tooth model described here reflects the in vivo environment with an adequate human root canal including variations in anatomic complexity. The proposed biofilm model will be useful for studies on antimicrobial efficacy of endodontic materials under clinically relevant conditions. Future studies investigating the effects of medicaments other than Ca(OH)2 on biofilms would benefit from the standardised biofilm tooth model as described in this study.

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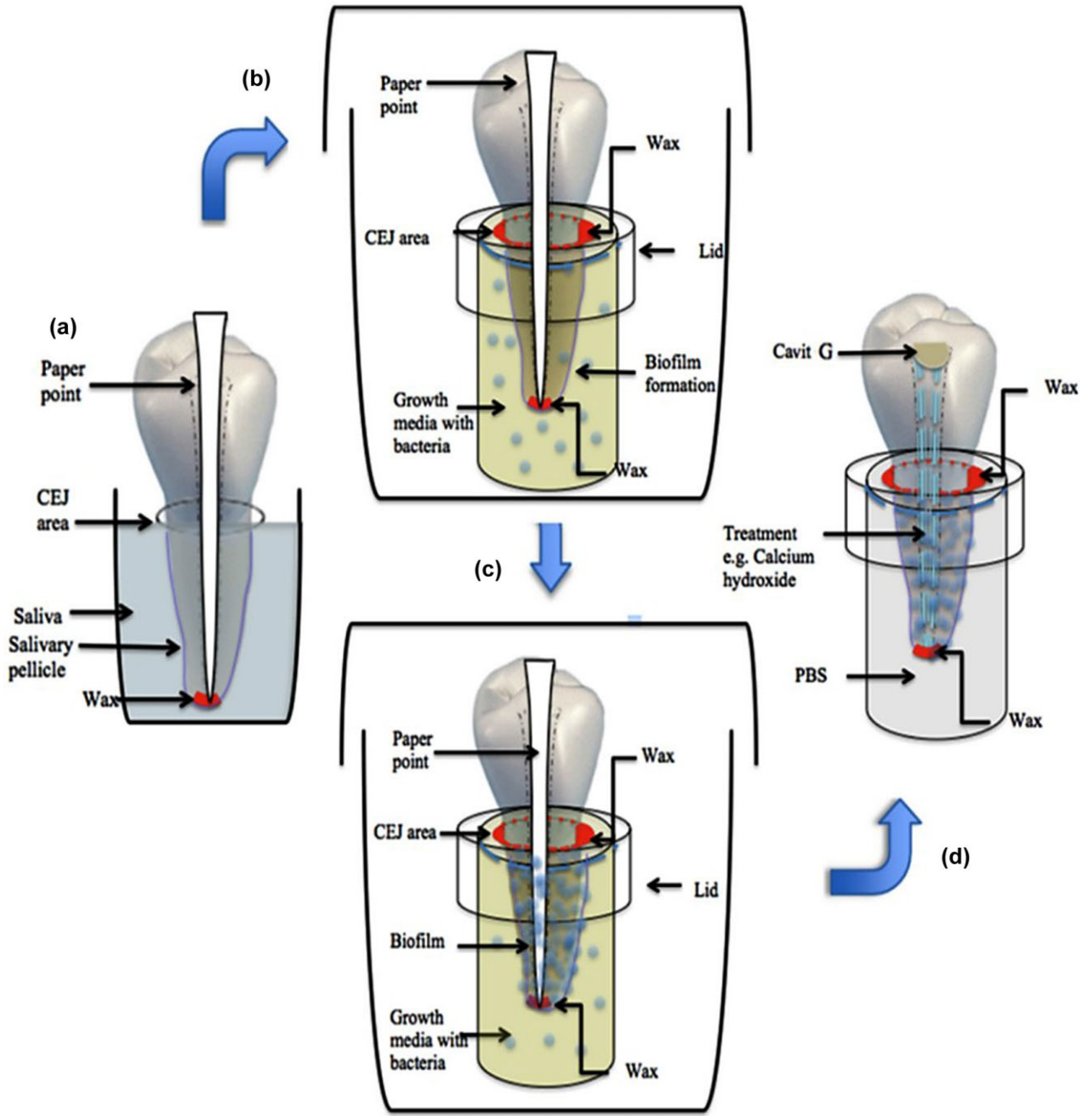

Figure 1 Diagrammatic representation of the three-dimensional fluorescent tooth model. (a) Root of tooth suspended in pooled fresh human saliva for 24 h to allow for the formation of pellicle. (b) Root of the tooth suspended in a 5-mL vial containing the bacterial growth media (Todd Hewitt broth) inoculated with 10 µL of overnight broth culture of *Streptococcus mutans*. (c) Root with sample set-up incubated for 4 days to allow for growth of bacterial biofilm. (d) Root of tooth suspended in phosphate-buffered saline (PBS) with bacterial biofilms on the surface of the root and calcium hydroxide medicament placed in the root canal and sealed using Cavit G. At this stage, the model is established and is considered as time zero. CEJ, cementoenamel junction.

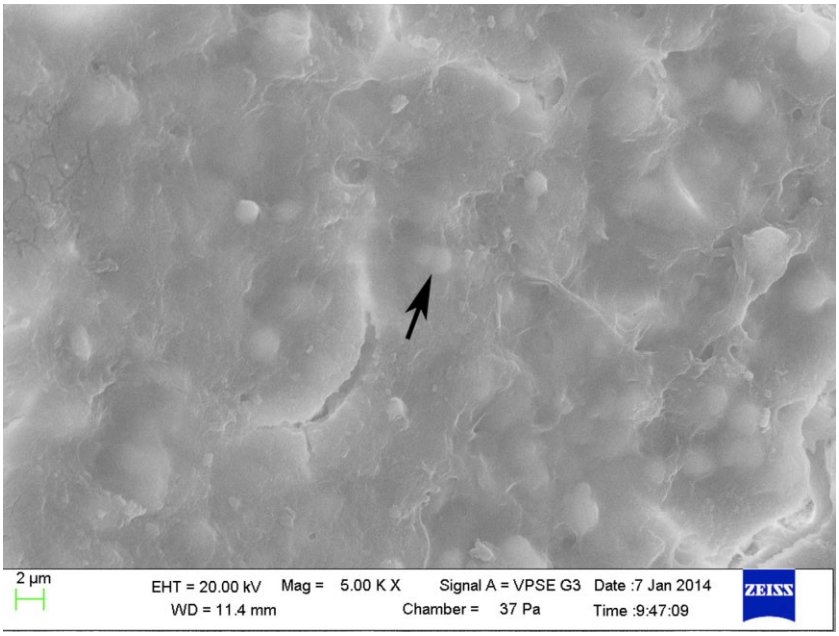

Figure 2 Scanning electron microscopy (SEM) image of for *Enterococcus faecalis* at 5000× magnification in the no treatment group. Note the presence of *E. faecalis* on the surface and embedded in the thick layer of biofilm at time zero.

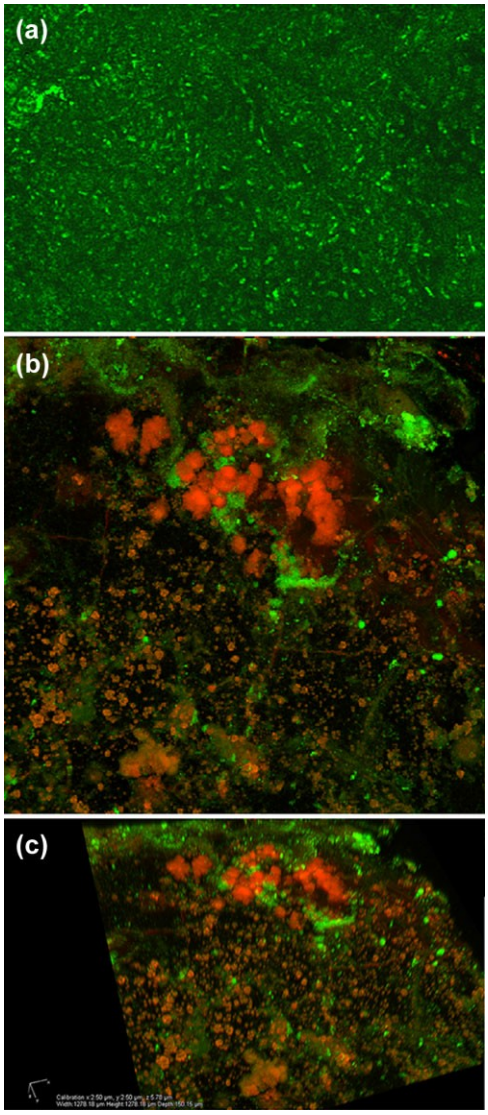

Figure 3 Panel showing proof of concept for the presence of *Enterococcus faecalis* bacterial biofilm on the external root surface (a) and bacterial kill following treatment (b and c). Note calibration properties of images captured with Nikon A1R laser scanning confocal microscope (c).

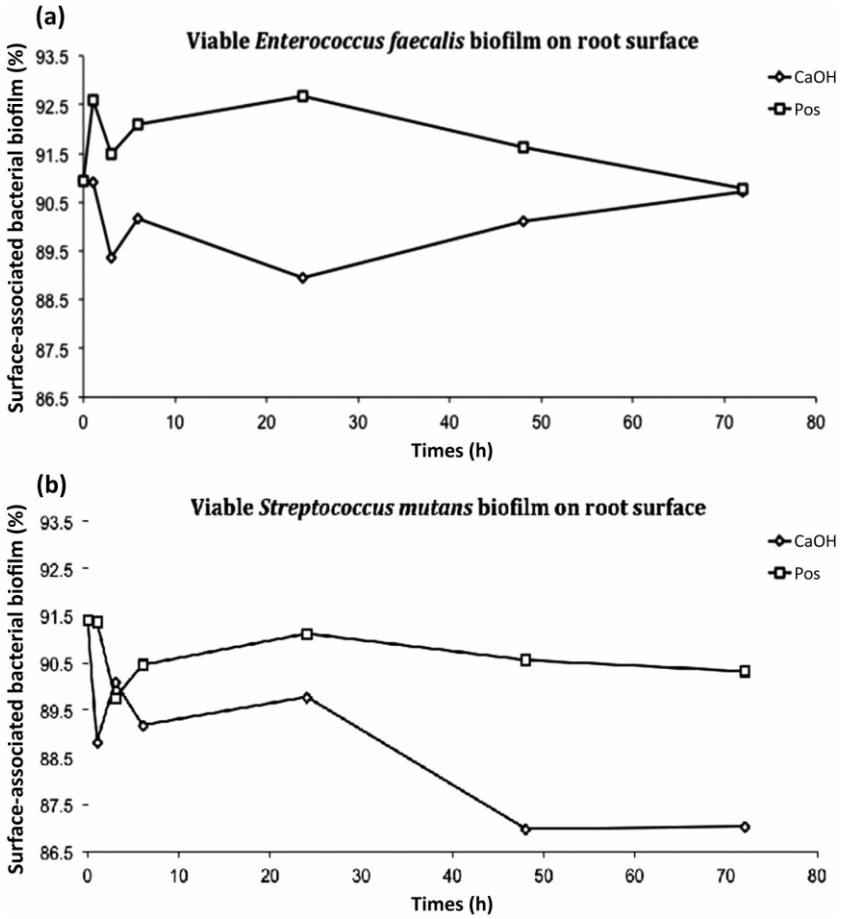

Figure 4 Top panel (a) show the viability of *Enterococcus faecalis* biofilm on the root surface following Ca(OH)2, calcium hydroxide treatment, compared with no treatment. Bottom panel (B) shows the viability of *Streptococcus mutans* biofilm on the apical region of root surface shown following Ca(OH)2 treatment, compared with no treatment.

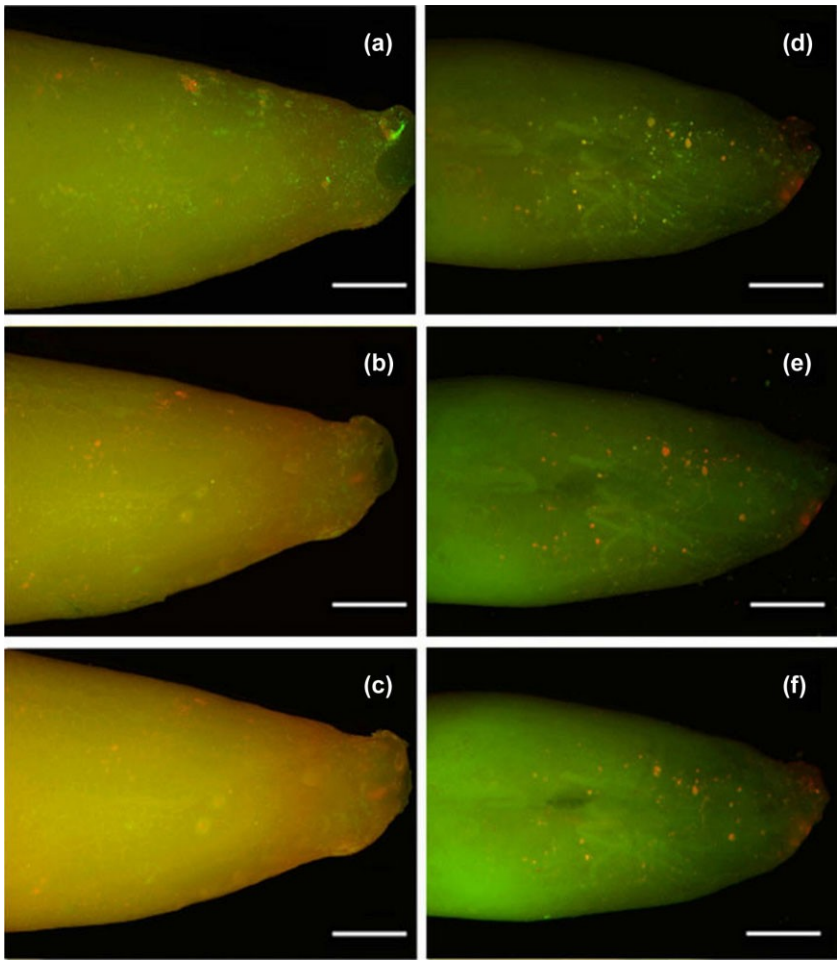

Figure 5 Panels a, b and c show *Streptococcus mutans* biofilm on the external surface of a tooth root treated with calcium hydroxide at time points 0 (a), 24 (b) and 72 h (c), respectively. Panels d, e and f show equivalent images for *Enterococcus faecalis*. Note the intensity of green (live bacteria) in panels d, e and f versus panels a, b and c. Scale bars are 1000 μm.

Table 1: Treatment of tooth samples

Bacteria	Treatment	Number of samples
Enterococcus faecalis	Positive control (no treatment)	7
	Calcium hydroxide	9
Streptococcus mutans	Positive control (no treatment)	7
	Calcium hydroxide	9